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# Modulation of $Ca^{2+}$ Channel Currents by a Novel Antidementia Drug *N*-(4-Acetyl-1-piperazinyl)-*p*-fluorobenzamide Monohydrate (FK960) in Rat Hippocampal Neurons

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**ABSTRACT**

*N*-(4-Acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate (FK960), a novel antidementia drug, has been demonstrated to ameliorate memory deficits in various experimental models of dementia. This drug selectively increases somatostatin release from hippocampal slices and augments long-term potentiation (LTP) in the CA3 area of the hippocampus. In the present study, the effects of FK960 on voltage-activated $Ca^{2+}$ channels were investigated in acutely isolated rat hippocampal neurons, using whole-cell patch-clamp technique to clarify the cellular mode of action of FK960. Application of somatostatin significantly reduced $Ca^{2+}$ currents via G protein-coupled signaling pathways. This inhibitory effect was significantly abolished by FK960 when applied in combination. In contrast, FK960 showed only modest inhibition on the reduction in $Ca^{2+}$ currents produced by baclofen, an agonist of $GABA_B$ receptor. Intracellular application of the protein kinase inhibitor H-7 did not alter somatostatin-induced inhibition and had no significant effect on blockade by FK960. In addition, application of FK960 alone produced modest but apparent increases in $Ca^{2+}$ currents without significant changes in the activation kinetics of the channels. The dose–response relationship on calcium current enhancement was bell-shaped with a maximum effect at 0.1 μM FK960, the same concentration as that for increasing on somatostatin release and CA3-LTP. These results show that FK960 reverses G protein-dependent inhibition of $Ca^{2+}$ currents by somatostatin in hippocampal neurons. Enhancement of $Ca^{2+}$ currents by FK960 may be due to its modulatory actions on $Ca^{2+}$ channels, rather than removal of G protein-inhibited tonic currents. Together, these mechanisms may be involved in the selective effects of FK960 on somatostatin release, excitatory transmission, and synaptic plasticity in the hippocampus.

Alzheimer's disease is the most common cause of dementia in the aged population and is accompanied by extensive neuron loss, particularly in the hippocampus and cerebral cortex, with concomitant progressive cognitive decline. We have recently discovered a novel antidementia drug, *N*-(4-acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate (FK960), which has been demonstrated to improve memory deficits in various experimental models of dementia such as passive avoidance, water-maze, and eight-arm radial maze tasks in rats (Yamazaki et al., 1996) and also in rhesus monkeys (Matsuoka and Aigner, 1997). Furthermore, we have also shown that FK960 selectively increases the release of somatostatin, but not of acetylcholine (ACh), γ-aminobutyric acid (GABA), noradrenaline, or serotonin from rat hippocampal slices (Inoue et al., 2001), and enhances long-term potentiation (LTP) in the mossy fiber-CA3 pathway of guinea pig hippocampal slices (Matsuoka and Satoh, 1998). LTP in the hippocampus is believed to be a component of learning and memory (Bliss and Collingridge, 1993). Several lines of evidence have suggested that brain somatostatin plays a vital role in regulating cognitive functions (Ohno et al., 1993; Epelbaum et al., 1994; Matsuoka et al., 1995). Clinical evidence has also shown that impairments of somatostatin-mediated neurotransmission in the brain are associated with dementia in Alzheimer's disease patients (Davies et al., 1980; Bissette and Myers, 1992). We have therefore suggested that FK960 acts on synaptic plasticity in the hippocampus through activation of somatostatinergic neurotransmission to exert its cognitive facilitating effects. However, the molecular mechanism responsible for the ability of FK960 to enhance somatostatin release is not fully understood.

The important regulatory mechanisms of neuronal $Ca^{2+}$ channels in multiple cellular functions such as transmitter release, synaptic excitability and transmission, hippocampal

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**ABBREVIATIONS:** ACh, acetylcholine; LTP, long-term potentiation; PTX, pertussis-toxin; GTPγS, guanosine 5'-O-(3-thio)triphosphate; H-7, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine; PKC, protein kinase C.

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LTP, and synapse formation have been extensively studied. The activation of voltage-activated $Ca^{2+}$ channels is regulated by neurotransmitters and a variety of intracellular second messenger pathways (Anwyl, 1991). Many neurotransmitters, including somatostatin, are known to inhibit $Ca^{2+}$ channels through G protein-coupled receptors and membrane-delimited pathways that may lead to autoreceptor-mediated inhibition of exocytotic release from presynaptic terminals (Dolphin 1995; Hille et al., 1995). Somatostatin selectively reduces N-type $Ca^{2+}$ channel currents in hippocampal and other central neurons possibly via the activation of pertussis-toxin (PTX)-sensitive Gi/Go proteins (Ikeda and Schofield, 1989; Ishibashi and Akaike, 1995; Viana and Hille, 1996). Moreover, the inhibitory effect of somatostatin on $Ca^{2+}$ channels results in the presynaptic inhibition of hippocampal synapse transmission (Boehm and Betz 1997).

To further understand the molecular basis for the ability of FK960 to enhance somatostatin release, in the present study, we investigated the effect of FK960 on voltage-dependent $Ca^{2+}$ channels and its interaction with somatostatin using whole-cell patch-clamp recording in acutely isolated rat hippocampal neurons. Here, we report that FK960 reverses the somatostatin-induced inhibition of $Ca^{2+}$ currents and that this effect seems to be mediated via a G protein-dependent mechanism. We also further demonstrated that FK960 has facilitatory actions on basal $Ca^{2+}$ channel currents in hippocampal neurons.

## Materials and Methods

**Cell Preparation.** Hippocampal neurons were acutely dissociated as described by Kay and Wong (1986) with slight modifications. Briefly, transverse hippocampal slices were prepared from 5- to 14-day-old male Wistar rats (Charles River Japan Inc., Yokohama, Japan). Slices were incubated at 30°C for 60 to 90 min in an oxygenated (100% $O_2$) solution containing 0.6 to 0.8 mg/ml trypsin (type I; Sigma-Aldrich, St. Louis, MO), 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM PIPES, and 25 mM glucose (pH 7.0). After rinsing with trypsin-free solution, slices were incubated at room temperature in an $O_2$ atmosphere. Before use, cells were triturated mechanically into individual cells with a fire-polished Pasteur pipette and were transferred to the recording chamber in Dulbecco's modified Eagle's medium. Visible pyramidal neurons with clear cellular membranes were chosen for whole-cell recording.

**Current Recording and Analysis.** Whole-cell recordings were performed using the conventional patch-clamp techniques (Hamill et al., 1981) with an Axopatch 200A amplifier (Axon Instruments, Union City, CA). Patch pipettes were fire-polished and had resistance of 2 to 4 MΩ when filled with the internal pipette solution containing 100 mM CsCl, 5 mM $MgCl_2$, 10 mM EGTA, 40 mM HEPES, 4 mM ATP-Tris, and 0.2 mM GTP-Tris (pH 7.3). The normal external solution for recording $Ca^{2+}$ channel currents consisted of 135 mM tetraethylammonium chloride, 10 mM $BaCl_2$, and 10 mM HEPES (pH 7.3). The recording chamber was continually perfused with the external solution with or without test drugs through gravity-fed flow pipes at a constant flow rate. Unless indicated otherwise, whole-cell inward currents were elicited every 10 s by depolarization to 0 mV from a holding potential of −80 mV. Currents were four-pole Bessel-filtered and digitized at 10 kHz with DigiData 1200 Interface. Data were acquired and leak subtracted using the P/4 protocol under the control of the pCLAMP (6.0) software (Axon Instruments) using a personal computer. All experiments were carried out at room temperature (21–23°C). All data are presented as mean ± S.E.M. ($n$ = number of cells in parentheses in the figures). Statistical analysis of data was performed using Student's $t$ test or Dunnett's multiple comparison test. A $p$ value less than 0.05 was considered significant.

**Pharmacological Materials.** FK960 was synthesized by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). A 10 mM stock solution of FK960 was prepared daily with distilled water and diluted in the external solution to the desired final concentrations just before use. Somatostatin, baclofen, GTPγS, pertussis toxin (PTX), and H-7 were obtained from Sigma-Aldrich. Somatostatin was dissolved in distilled water and frozen stored until use. Both GTPγS and H-7 were dissolved in the pipette solution immediately before use. GTP in the standard pipette solution was omitted when GTPγS was included.

## Results

**FK960 Disrupts Somatostatin-Induced Inhibition of $Ca^{2+}$ Currents.** $Ba^{2+}$ was used as the charge carrier for the recording of $Ca^{2+}$ currents in hippocampal neurons. Under whole-cell patch-clamp configuration, high-voltage-activated inward $Ba^{2+}$ currents were completely blocked by external application of $Cd^{2+}$, indicating that they pass through $Ca^{2+}$ channels. Previous results have demonstrated that somatostatin selectively inhibits N-type $Ca^{2+}$ channels in hippocampal neurons because these currents are sensitive to ω-conotoxin GVIA (Ishibashi and Akaike, 1995). Figure 1 illustrates the effect of FK960 on somatostatin-induced inhibition of $Ca^{2+}$ currents in isolated rat hippocampal neurons. External application of somatostatin (0.1 μM) rapidly reduced the peak current amplitude and slowed the activation kinetics of $Ca^{2+}$ currents (Fig. 1A). The inhibition by somatostatin was reversible after removal of somatostatin and was reproduced by repeated application of somatostatin with slight desensitization (Fig. 1B). In cells perfused with both somatostatin (0.1 μM) and FK960 (0.1 μM) concomitantly, however, the inhibition of $Ca^{2+}$ currents by somatostatin was not observed. FK960 completely blocked the somatostatin-induced inhibition and eliminated the kinetic slowing. In contrast to the mean inhibition of 23.03 ± 1.80% ($n$ = 12) produced by somatostatin in control conditions, there was only 2.17 ± 3.91% ($n$ = 8) reduction in $Ca^{2+}$ currents in the presence of FK960 (Fig. 1C).

Considering that $Ca^{2+}$ channels are regulated by a variety of neurotransmitters, we further investigated the effect of FK960 on inhibition of $Ca^{2+}$ channels mediated by $GABA_B$ receptor. As shown in Fig. 2, application of a $GABA_B$ receptor agonist baclofen (25 μM) reduced the peak currents with an average 16.33 ± 2.88% ($n$ = 7) inhibition in control condition and 11.38 ± 2.63% ($n$ = 7) inhibition in the presence of 0.1 μM FK960. Although FK960 tended to reduce baclofen-induced inhibition, this effect was partial and was not statistically significant compared with baclofen alone-treated group, in contrast to the combination with somatostatin. These results suggest that FK960 selectively disrupts inhibition of $Ca^{2+}$ currents produced by somatostatin.

**G Protein-Mediated Inhibition by Somatostatin.** It is well demonstrated that voltage-dependent N-type $Ca^{2+}$ channel activity is regulated by a G protein-coupled membrane-delimited pathway (Hille et al., 1995; Dolphin 1995) and the G protein-mediated inhibition of $Ca^{2+}$ currents can be relieved by a large degree of depolarization (Bean, 1989; Ikeda, 1991). To test the voltage-dependent facilitation of current inhibition, currents were observed after a stronger depolarizing step to +80 mV. Under control conditions, the

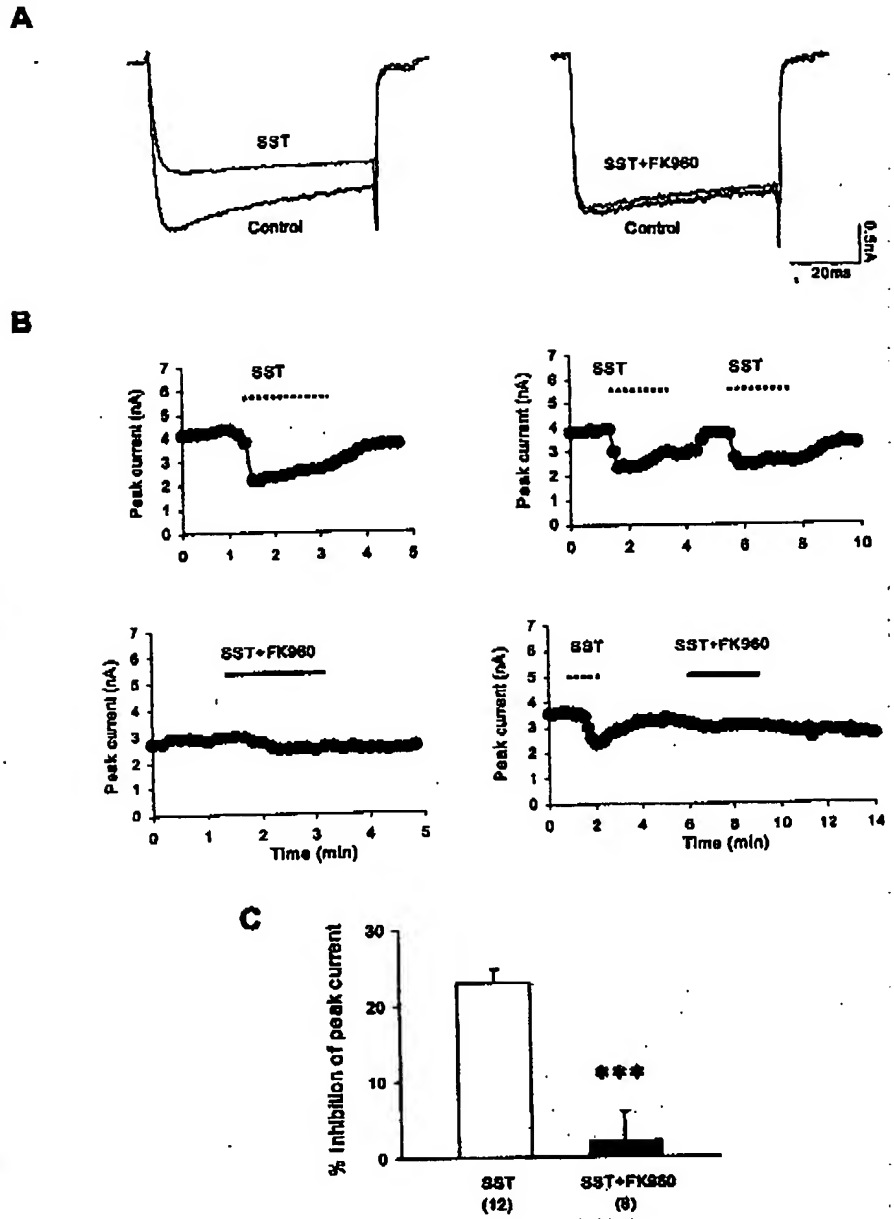


Fig. 1. Effect of FK960 on somatostatin-induced inhibition of $Ca^{2+}$ currents in hippocampal neurons. Currents were evoked every 10 s by a depolarization to 0 mV from a holding potential of −80 mV. A, superimposed current traces were obtained in control external solution and 0.1 μM somatostatin without or with 0.1 μM FK960. B, time course of the peak current in application of somatostatin (0.1 μM) in the presence or absence of 0.1 μM FK960. C, pooled results of peak current inhibited by somatostatin in the absence or presence of FK960. ***, $p < 0.001$; statistically significant compared to somatostatin alone group (by Student's $t$ test). SST; somatostatin.

depolarizing prepulse did not produce characteristic facilitation of the $Ca^{2+}$ currents. This implied the lack of tonic inhibition of $Ca^{2+}$ currents by G proteins in these cells that has been shown in several neuronal systems, even in the absence of neurotransmitters (Ikeda, 1991; Kasai, 1991). As shown in Fig. 3A, somatostatin-induced inhibition of $Ca^{2+}$ currents was mostly but not completely relieved, and the altered current kinetics was eliminated after the depolarizing prepulse, suggesting that voltage-dependent components reliving in somatostatin action. There was still a small portion of currents remaining after the prepulse in these cells. This may be due to the voltage protocol used here that resulted in incomplete recovery from somatostatin inhibition. In contrast, the application of FK960 abolished somatostatin inhibition before and after the prepulse was applied. Furthermore, FK960 restored the relief of the resting currents after the prepulse in these cells. These results suggest that FK960 modulates voltage-dependent inhibition of $Ca^{2+}$ channels by somatostatin.

Line of evidence has shown that many neurotransmitters, including somatostatin, inhibit voltage-activated $Ca^{2+}$ channels through direct activation of inhibitory G proteins (Shapiro and Hille, 1993; Hille et al., 1995; Zhang et al., 1996; Herlitze et al., 1996). In the present study, we used GTPγS, a nonhydrolyzable analog of GTP, to characterize the G protein-dependent inhibition by somatostatin. When 100 μM GTPγS was intracellularlly present in the pipette solution, the basal currents were reduced before somatostatin application (data not shown). As shown in Fig. 3B, the mean percentage of inhibition by somatostatin was 22.49 ± 1.96%

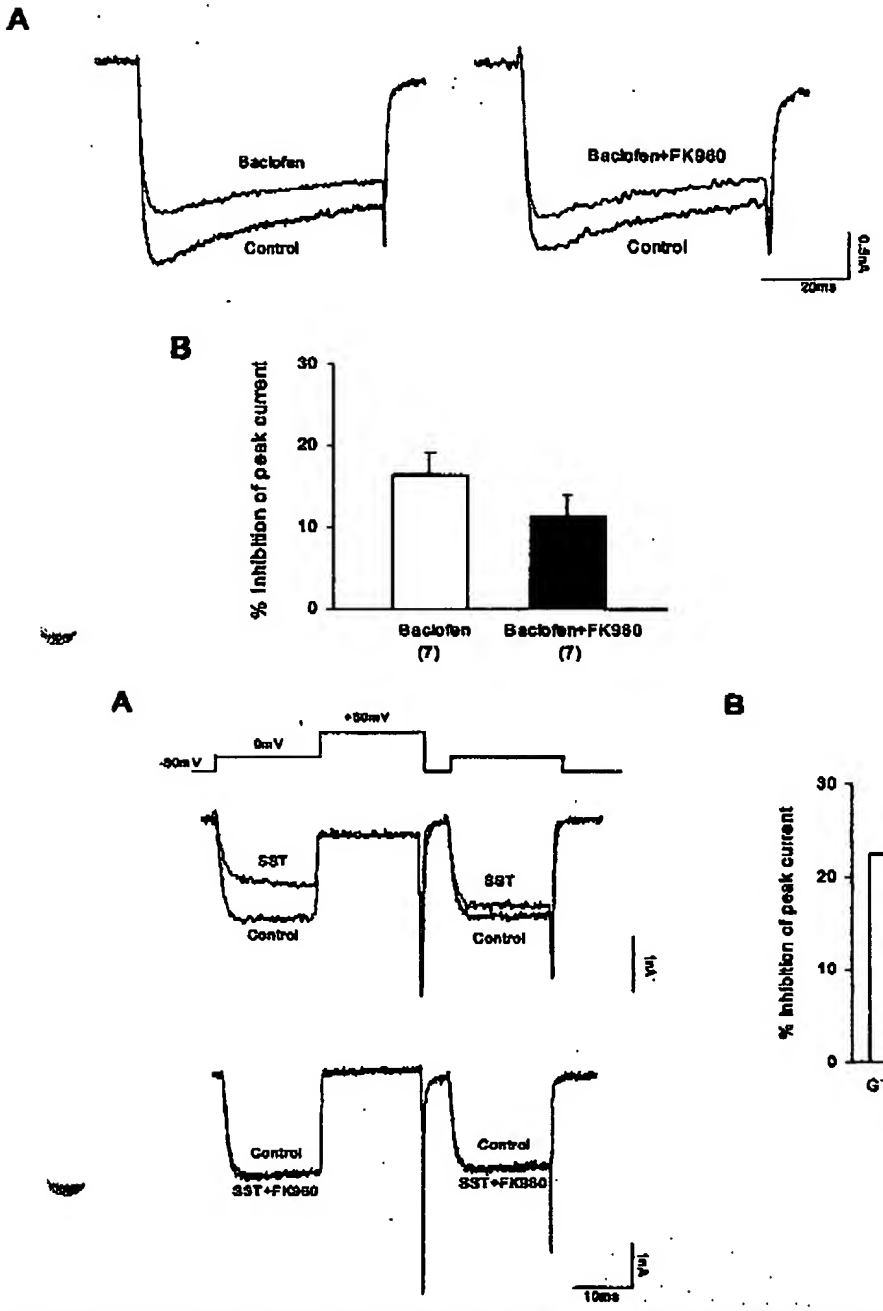


Fig. 2. Effect of FK960 on baclofen-induced inhibition of $Ca^{2+}$ currents in hippocampal neurons. A, superimposed current traces were obtained in control external solution and 25 $\mu M$ baclofen without or with 0.1 $\mu M$ FK960. B, pooled results show the peak current inhibition produced by baclofen and concomitant application with FK960. No statistical significant change was observed between two groups (by Student's $t$ test).




Fig. 3. Involvement of G proteins in the action of somatostatin. A, effect of prepulse facilitation on somatostatin-induced inhibition. Currents were evoked by the voltage protocol as shown above. Superimposed current traces were obtained in control external solution and 0.1 $\mu M$ somatostatin without (top traces) or simultaneously with 0.1 $\mu M$ FK960 (bottom traces). SST, somatostatin. B effects of intracellular GTP$\gamma$S and PTX treatment. Peak currents were reduced by the intracellular application of 100 $\mu M$ GTP$\gamma$S or by pretreatment of PTX. Both of them blocked significantly the somatostatin-induced inhibition. Pooled results show the peak current inhibition by somatostatin in the absence or presence of FK960. **, $p < 0.01$; statistically significant compared to somatostatin alone group (by Student's $t$ test).

($n = 9$) in the control condition but was largely reduced to $4.51 \pm 4.20\%$ ($n = 4$) when GTP$\gamma$S was added in the pipette solution ($p < 0.01$). For PTX pretreatment, currents were obtained after incubation in culture medium containing high concentration of PTX (25 $\mu g$/ml) at 35°C for more than 1 h (Beech et al., 1992). Application of somatostatin had an inhibition of $2.18 \pm 5.6\%$ ($n = 3$), compared with $22.73 \pm 2.45\%$ ($n = 7$) inhibition obtained without PTX treatment ($p < 0.01$). Together, both of GTP$\gamma$S and PTX pretreatment significantly eliminated somatostatin-induced inhibition of $Ca^{2+}$ currents, suggesting that PTX-sensitive G proteins are involved in somatostatin-induced inhibition of $Ca^{2+}$ channel currents.

**Involvement of Protein Kinase Activation.** Several protein kinases are implicated in neurotransmitter receptor-mediated modulation of $Ca^{2+}$ channels by phosphorylation of transmitter receptors themselves, the associated G proteins,

and functional domains of $Ca^{2+}$ channel subunits (Ahlijanian et al., 1991; Swartz, 1993; Stea et al., 1995; Zamponi et al., 1997; Hamid et al., 1999; Cooper et al., 2000). To test whether protein kinase activation is involved in the effects of FK960 and somatostatin, we used a broad protein kinase inhibitor H-7 by intracellular application. Figure 4 illustrates the effects on $Ca^{2+}$ currents by somatostatin and FK960 with 50 μM H-7 in the pipette solution. In the intracellular presence of H-7, somatostatin reduced the peak current amplitudes with mean inhibition of 23.29 ± 4.33% ($n$ = 8), similar to control conditions. FK960 again significantly abolished the somatostatin-induced inhibition of $Ca^{2+}$ currents in the presence of H-7 treatment; however, the magnitude of the reduction by FK960 (mean somatostatin inhibition of 6.98 ± 2.73%; $n$ = 8) was slightly smaller compared with that in control cells (Fig. 1C). Basal $Ca^{2+}$ currents were unchanged by intracellular application of H-7 (data not shown). These results show that blocking of protein kinase activity by H-7 in our cell preparation has no significant effects on somatostatin receptor-mediated inhibition of $Ca^{2+}$ channels.

**FK960 Enhances the Basal $Ca^{2+}$ Currents in Hippocampal Neurons.** In addition to its modulating actions on somatostatin-induced inhibition of $Ca^{2+}$ currents, we found that external application of FK960 reversibly enhances $Ca^{2+}$ currents under the basal condition in some hippocampal neurons. Figure 5 illustrates the increase in the amplitude of peak currents after application of FK960. Application of FK960 did not alter the kinetics of the channels but increased the peak currents at most test potentials without measurable voltage dependence (Fig. 5B). Enhancement of $Ca^{2+}$ currents by FK960 displayed a bell-shaped concentration dependence that is similar to that seen in our previous pharmacological studies (Matsuoka and Satoh, 1998; Inoue et al., 2001). As illustrated in Fig. 5C, application of 0.01 to 1 μM FK960 significantly enhanced the basal currents, and the maximal effect was obtained at a concentration of 0.1 μM FK960. Differences between 0.1 μM and other doses of FK960 were not statistically significant.

## Discussion

Regulation of neuronal voltage-activated $Ca^{2+}$ channels by neurotransmitters and intracellular signaling pathways is an important step in the control of neurotransmitter release, synaptic transmission, and neuronal plasticity. In the present study, we have determined the effect of a novel anti-dementia drug FK960 on voltage-activated $Ca^{2+}$ channels in isolated rat hippocampal neurons. Our results demonstrate for the first time that FK960 modulates the G protein-mediated inhibitory effect of somatostatin on $Ca^{2+}$ channels and, furthermore, enhances the basal $Ca^{2+}$ currents in hippocampal neurons.

It has been suggested that somatostatin receptors inhibit N-type $Ca^{2+}$ channels via PTX-sensitive G proteins through a direct membrane-delimited model (Shapiro and Hille, 1993; Hille et al., 1995; Zhang et al., 1996). Somatostatin-induced inhibition of $Ca^{2+}$ currents in these isolated hippocampal neurons is mediated by activation of G proteins because PTX as well as GTPγS eliminated this inhibition. In addition, prepulse facilitation relieved most of the current reduction by somatostatin, a characteristic form of inhibition occurring via a direct interaction of the channel and G protein (Hille et al., 1995). Importantly, FK960 disrupted G protein-dependent inhibition of $Ca^{2+}$ currents by somatostatin. Inhibitory effect of FK960 was more robust than that induced by depolarizing prepulse, which resulted in the recovery from somatostatin inhibition of $Ca^{2+}$ currents. Somatostatin-induced inhibition of $Ca^{2+}$ channels has been suggested to be one of the mechanisms underlying presynaptic inhibitory control of transmitter release (Boehm and Betz, 1997). Therefore, the ability of FK960 to modulate the inhibitory effect of somatostatin on $Ca^{2+}$ channels may contribute to its ability to selectively enhance somatostatin release and somatostatinergic transmission in hippocampal slices (Inoue et al., 2001).

In the present study, we found that basal $Ca^{2+}$ channel currents in hippocampal neurons were enhanced by FK960. Compared with its obvious effect on somatostatin-induced depression of $Ca^{2+}$ currents, the enhancement of basal $Ca^{2+}$ currents produced by FK960 was more modest, and was not

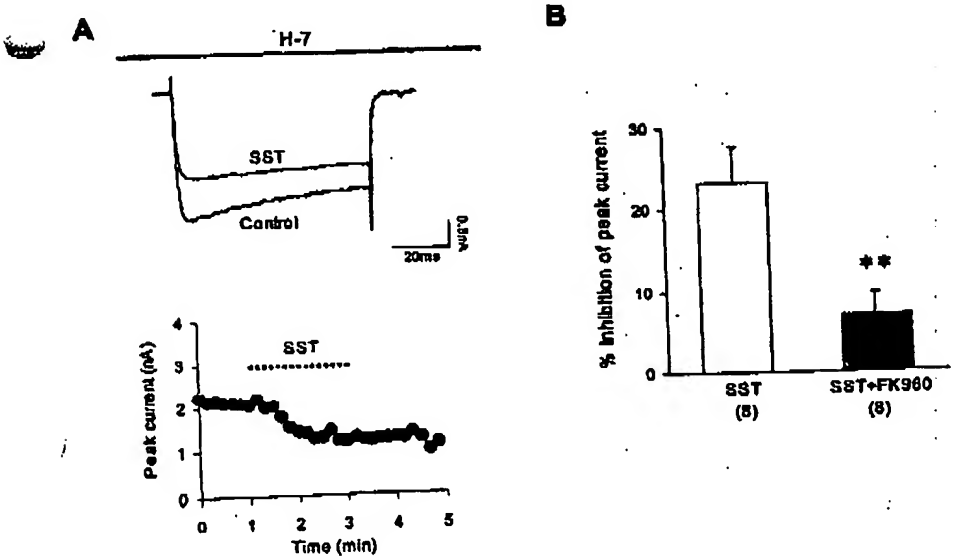


Fig. 4. Effect of protein kinase inhibitor H-7 on somatostatin-induced inhibition of $Ca^{2+}$ currents and on the blockade by FK960. A, superimposed inward currents were recorded before and after application of 0.1 μM somatostatin in the intracellular presence of 50 μM H-7 (top trace). Time courses of peak current recorded under application of 0.1 μM somatostatin (bottom trace). B, pooled results of peak current inhibited by somatostatin in the absence or presence of FK960. **, $p$ < 0.01, statistically significant compared with somatostatin-alone group (by Student's $t$ test). SST; somatostatin.

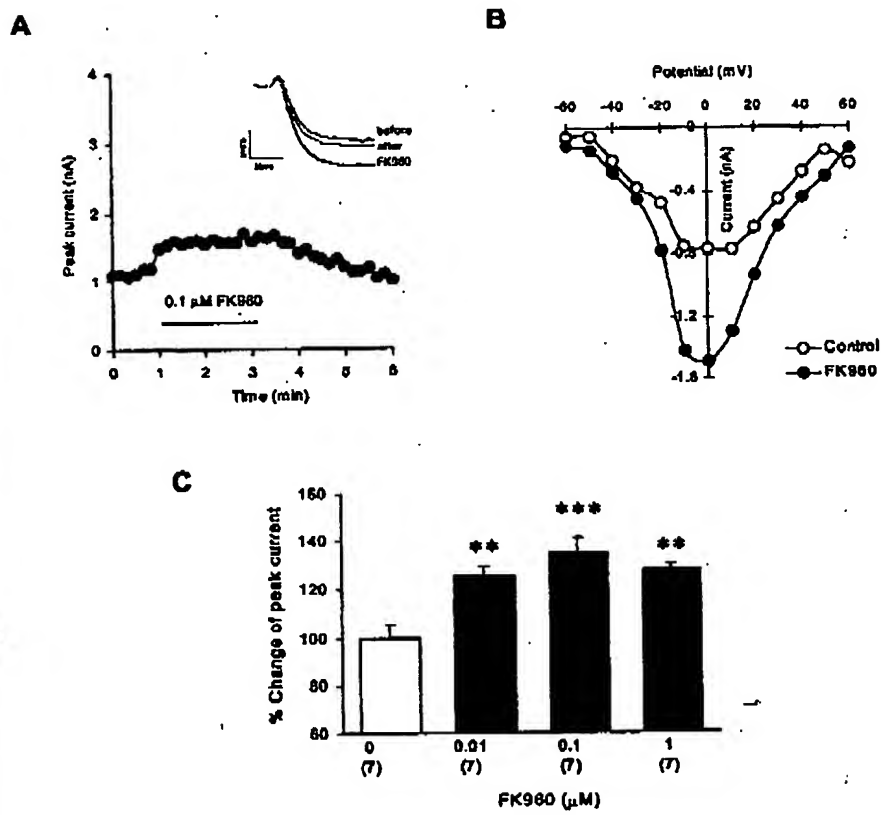


Fig. 5. Enhancement of the basal $Ca^{2+}$ currents by FK960. A, time course of enhancement of peak currents by 0.1 μM FK960. Inset shows superimposed currents recorded before, during, and after FK960 application. B, peak current-voltage relationship in the control condition and application of FK960 in the same cell. C, concentration-effect relationship for the enhancement of $Ca^{2+}$ currents produced by FK960. Pooled results show the percentage changes of peak currents in the absence (set as 100%) and presence of FK960. **, $p < 0.01$; ***, $p < 0.001$, statistically significant compared to control group (by analysis of variance followed by Dunnett's comparison test).

observed in all cells. Although several types of voltage-activated $Ca^{2+}$ channels have been identified in the hippocampal neurons, it has been suggested that N-type channels predominantly contribute to the transmitter-stimulated synaptic transmission (Wheeler et al., 1994). Further study is necessary to determine which type of $Ca^{2+}$ channel current is enhanced by FK960. The FK960-induced increase in basal $Ca^{2+}$ channel currents is unlikely to be a consequence of removal of G protein-mediated tonic inhibition, as observed in some neurons (Dolphin, 1995), because the rebound current facilitation by a large depolarization pulse was not observed in these cells. The facilitatory effect of FK960 on $Ca^{2+}$ current demonstrated a bell-shaped concentration-response relationship comparable with our previous studies on somatostatin release and CA3-LTP enhancement, indicating the phenomenon might share common cellular mechanisms.

Voltage-activated $Ca^{2+}$ channel activities are regulated by a variety of neurotransmitter and intracellular signaling pathways (Hille et al., 1995; Dolphin, 1995). Therefore, the modulation of $Ca^{2+}$ channels by FK960 could be mediated at the level of neurotransmitter receptor, G proteins, $Ca^{2+}$ channels, or other intracellular signaling pathways. Our previous studies have shown that FK960 does not bind to somatostatin receptors or a number of other neurotransmitter receptors (unpublished observations), although the possibility of an unknown protein component associated to its action could not be ruled out. Our present results demonstrated that FK960 disrupted G protein-dependent inhibition by somatostatin and enhanced the basal $Ca^{2+}$ current, further suggesting that the modulation of FK960 is not in the level of somatostatin receptors activation and seems to be involved in the direct interaction between G protein and $Ca^{2+}$ channels. Recently, an occluded inhibition of $Ca^{2+}$ channels by activation of two types of transmitter receptor by opioid agonists and somatostatin has been investigated (Polo-Parada and Pilar, 1999).

Protein kinases are important for regulation of neuronal $Ca^{2+}$ channel activity and have been shown to directly phosphorylate the channel subunit/G protein complex (Swartz, 1993; Hamid et al., 1999; Cooper et al., 2000). In central neurons, activation of PKC has been found to augment $Ca^{2+}$ currents (Swartz et al., 1993; Stea et al., 1995; Hamid et al., 1999) and disrupt G protein-dependent inhibition of $Ca^{2+}$ channels (Swartz, 1993; Zamponi et al., 1997; Barrett and Rittenhouse, 2000). Furthermore, PKC activators block the inhibition of $Ca^{2+}$ currents induced by somatostatin in rat hippocampal neurons (Ishibashi and Akaike, 1995). In the present study, intracellular application of protein kinase inhibitor H-7 had no effect on somatostatin-induced inhibition of $Ca^{2+}$ currents. In addition, H-7 slightly reduced FK960's modulation of somatostatin inhibition, but the effect was not significant. Considering that H-7 is known to be relatively more effective on cAMP (or cGMP)-dependent kinase activation than on PKC activation, however, we could not rule out the possibility of PKC-mediated regulation involved in the mechanism of action of FK960. Further studies with highly selective PKC modulators are required to clarify the contribution of intracellular phosphorylation pathways to the mechanism of FK960 action.

It is conceivable that modulation by FK960 of somatostatin-mediated inhibition of $Ca^{2+}$ channels may be responsible for its enhancement of somatostatin release from hippocampal slices. Many studies have demonstrated the existence of negative feed-

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back regulation via presynaptic G protein-coupled receptors (autoreceptors) as well as voltage-activated $Ca^{2+}$ channels, leading to inhibition of transmitter release (Dolphin, 1995; Hille et al., 1995; Takahashi et al., 1996). Indeed, somatostatin release from nerve terminals is $Ca^{2+}$-dependent and is modulated through activation of an autoreceptor located on presynaptic terminals (Iversen et al., 1978; Fontana et al., 1996; Helboe et al., 1998). Furthermore, somatostatin selectively inhibits the N-type $Ca^{2+}$ channel among diverse subtypes of channels in isolated hippocampal neurons (Ishibashi and Akaike, 1995). It has also been reported that somatostatin inhibits excitatory neurotransmission via presynaptic receptors, by inhibition of downstream of $Ca^{2+}$ entry at rat hippocampal synapses (Boehm and Betz, 1997). It will be important to determine whether FK960 acts exclusively on somatostatin-mediated inhibition, or whether it can disrupt the inhibition produced by other transmitters, which involve G protein-coupled receptors. In the present study, interestingly, FK960 showed only modest inhibition on the reduction in $Ca^{2+}$ currents produced by an activation of $GABA_B$ receptor, suggesting that FK960's action is not general for G protein-coupled receptors and could be selective for somatostatin receptor over other class of G protein-coupled receptors. Somatostatin-containing neurons are abundant in the hippocampus, and, although they often colocalize or functionally interact with other neurotransmitters such as GABA or ACh, they might regulate $Ca^{2+}$ channels through different G protein pathways (Shapiro and Hille, 1993; Hille et al., 1995). In our studies on neurotransmitter release in rat hippocampal slices, we found that FK960 had no significant effect on ACh, GABA, noradrenaline, and serotonin release (Inoue et al., 2001). We therefore propose that FK960 could exert selective facilitatory actions on somatostatin release from hippocampal nerve terminals, as a consequence of blockade of the interplay between the somatostatin autoreceptor, inhibitory G protein, and (possibly N-type) $Ca^{2+}$ channels.

In conclusion, we have demonstrated for the first time that the novel antidementia drug FK960 reverses the inhibitory effect of somatostatin on $Ca^{2+}$ channels and enhances the basal activity of $Ca^{2+}$ currents in rat hippocampal neurons. These cellular mechanisms may explain the unique mode of action of FK960 and may further provide new insights on the molecular basis for the understanding the control of neuropeptide release from presynaptic terminals.

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# Cross-talk between G-protein and Protein Kinase C Modulation of N-type Calcium Channels Is Dependent on the G-protein β Subunit Isoform*

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**The modulation of N-type calcium current by protein kinases and G-proteins is a factor in the fine tuning of neurotransmitter release. We have previously shown that phosphorylation of threonine 422 in the $\alpha_{1B}$ calcium channel domain I-II linker region resulted in a dramatic reduction in somatostatin receptor-mediated G-protein inhibition of the channels and that the I-II linker consequently serves as an integration center for cross-talk between protein kinase C (PKC) and G-proteins (Hamid, J., Nelson, D., Spaetgens, R., Dubel, S. J., Snutch, T. P., and Zamponi, G. W. (1999) *J. Biol. Chem.* 274, 6195–6202). Here we show that opioid receptor-mediated inhibition of N-type channels is affected to a lesser extent compared with that seen with somatostatin receptors, hinting at the possibility that PKC/G-protein cross-talk might be dependent on the G-protein subtype. To address this issue, we have examined the effects of four different types of G-protein β subunits on both wild type and mutant $\alpha_{1B}$ calcium channels in which residue 422 has been replaced by glutamate to mimic PKC-dependent phosphorylation and on channels that have been directly phosphorylated by protein kinase C. Our data show that phosphorylation or mutation of residue 422 antagonizes the effect of $G\beta_1$ on channel activity, whereas $G\beta_2$, $G\beta_3$, and $G\beta_4$ are not affected. Our data therefore suggest that the observed cross-talk between G-proteins and protein kinase C modulation of N-type channels is a selective feature of the $G\beta_1$ subunit.**

The modulation of calcium channel activity by activation of intracellular messenger pathways is a key mechanism for fine tuning calcium entry into neurons. For example, the activation of protein kinase C has been shown to mediate an up-regulation of N-type calcium currents in intact neurons (1, 2) and in transient expression systems (3, 4). In contrast, the direct 1:1 binding of G protein βγ subunits to the domain I-II linker region of N-type, P/Q-type, and possibly R-type calcium channels results in a depression of current activity (5–8) (reviewed in Refs. 9 and 10), which can be reversed by strong membrane depolarization (10–12). Different types of calcium channels are modulated by G-proteins to different extents, such that N-type channels are typically inhibited more effectively than P/Q-type channels (13–16). There is also increasing evidence that the degree of inhibition is dependent on the G-protein β subunit species (16–18). Finally, it has been shown that protein kinase C-dependent phosphorylation of the N-type calcium channel $\alpha_1$ subunit antagonizes receptor-mediated G-protein inhibition of the channel (1, 2, 12, 19). This phenomenon (termed PKC[1]/G-protein cross-talk) appears to be mediated by a single threonine residue (Thr-422) in the $\alpha_{1B}$ domain I-II linker region (4). For somatostatin receptor-induced G-protein inhibition of N-type calcium channels, mutation of Thr-422 to glutamic acid mimics the antagonistic effect of protein kinase C on G protein inhibition, whereas a switch to alanine precludes the occurrence of PKC/G-protein cross-talk (4).

Here we have examined the dependence of PKC/G-protein cross-talk on the nature of the G protein β subunit isoform. Using transient expression of either wild type or mutant (T422E) N-type calcium channels in combination with various G-protein β subunit isoforms ($G\beta_1$, $G\beta_2$, $G\beta_3$, and $G\beta_4$), we show that the effect of only the $G\beta_1$ isoform is reduced in the "permanently phosphorylated" mutant N-type channel. PKC/G-protein cross-talk thus appears to be a selective feature of the $G\beta_1$ subunit isoform. In view of the notion that different types of G-protein-coupled receptors may combine with specific subsets of $G\alpha\beta\gamma$ combinations (20), this may suggest that the extent of cross-talk occurring in intact neurons could be dependent on the type of neurotransmitter involved, thus providing a mechanism for the fine tuning of calcium homeostasis.

## EXPERIMENTAL PROCEDURES

*Calcium Channel and G-protein cDNAs*—The calcium channel cDNA constructs ($\alpha_{1B}$, $\alpha_{1B}$(T422E), $\beta_{1b}$, $\alpha_2$-δ) were the same as those discussed previously in Hamid *et al.* (4). Wild type constructs were kindly donated by Dr. T. P. Snutch. The cloning of the various Gβ-subunits is described

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[1] The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DAMGO, [D-Ala², *N*-methyl-Phe⁴, Gly⁵-ol]-enkephalin.

in detail by Arnot *et al.* (16). cDNA encoding for green fluorescent protein (EGFP) was purchased from CLONTECH.

*Transient Transfection into tsa-201 Cells*—Human embryonic kidney tsa-201 cells were grown in standard Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.5 mg/ml penicillin/streptomycin. Cells were grown at 37 °C to 85% confluency, split with trypsin-EDTA, and plated on glass coverslips at 5–10% confluency about 12 h before transfection. Immediately before transfection, the medium was replaced with fresh Dulbecco's modified Eagle's medium, and a standard calcium phosphate protocol was used to transiently transfect the cells with cDNA constructs encoding for mutant and wild type calcium channel $\alpha_1$, $\beta_{1b}$, and $\alpha_2$-$\delta$ subunits and green fluorescent protein as an expression marker and as appropriate with G-protein subunits or the $\mu$-opioid receptor. After 12 h, cells were washed with fresh medium, allowed to recover for 12 h, and then incubated at 28 °C in 5% $CO_2$ for 1–3 days prior to recording.

*Electrophysiology*—The exact whole cell recording procedures have been described in detail previously (16). The external recording solution was comprised of 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 65 mM CsCl (pH 7.2), recording pipettes were filled with 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, 9 mM HEPES (pH 7.2) and showed typical resistances of 2 to 4 megaohms. For experiments involving opioid receptor activation, the intracellular solution was supplemented with 40 $\mu$M GTP. DAMGO (purchased from RBI) was dissolved in water at a stock concentration of 2 mM, diluted into the recording solution at a final concentration of 1 $\mu$M, and perfused directly into the vicinity of the cells via a gravity-driven microperfusion system. Typically, cells were held at −100 mV and currents were elicited upon depolarizations to various test potentials.

Tonic G-protein inhibition of the channels was assessed by application of strong depolarizing prepulses (to +150 mV) followed by a test depolarization to +20 mV (see Refs. 8 and 16). The time course of development of prepulse relief was determined by varying the duration of the prepulse ($\Delta t1$) while leaving the duration between the prepulse and the test pulse constant at 5 ms (see Fig. 2). The reinhibition kinetics were determined by applying a 50-ms prepulse, followed by a test pulse spaced from the prepulse at variable intervals ($\Delta t2$). The total degree of prepulse relief was obtained by extrapolating the exponential decay of the prepulse effect back to time $t2 = 0$ (see Ref. 16), thus allowing us to assess the degree of prepulse relief without contamination from reinhibition. For the experiment in Fig. 2, the data were obtained at a fixed interval of 4 ms between the prepulse and the test pulse.

For experiments involving PKC-dependent phosphorylation of the channels, the phorbol ester PMA was dissolved in $Me_2SO$ at a stock concentration of 2 mM, diluted into the recording solution at a final concentration of 30 nM, and perfused directly onto the cell with a gravity-driven microperfusion system.

*Western Blot Analysis*—Western blots on cell lysates generated from transfected or sham-transfected tsa-201 cells were carried out as described in detail by Jarvis *et al.* (21). Antibodies to G protein $\beta$ subunits were purchased from Santa Cruz Laboratories (anti-G$\beta_2$ and anti-G$\beta_3$) and from Transduction Laboratories (anti-G$\beta_{1/4}$). Immunoblots were subjected to chemiluminescence analysis using ECL plus (Amersham Pharmacia Biotech) and detected on film.

*Data Analysis and Statistics*—Data were analyzed using Clampfit software. Preparation of figures and statistical analysis was performed via SigmaPlot (Jandel Scientific). *Error bars* are standard errors, $p$ values reflect Student's $t$ tests.

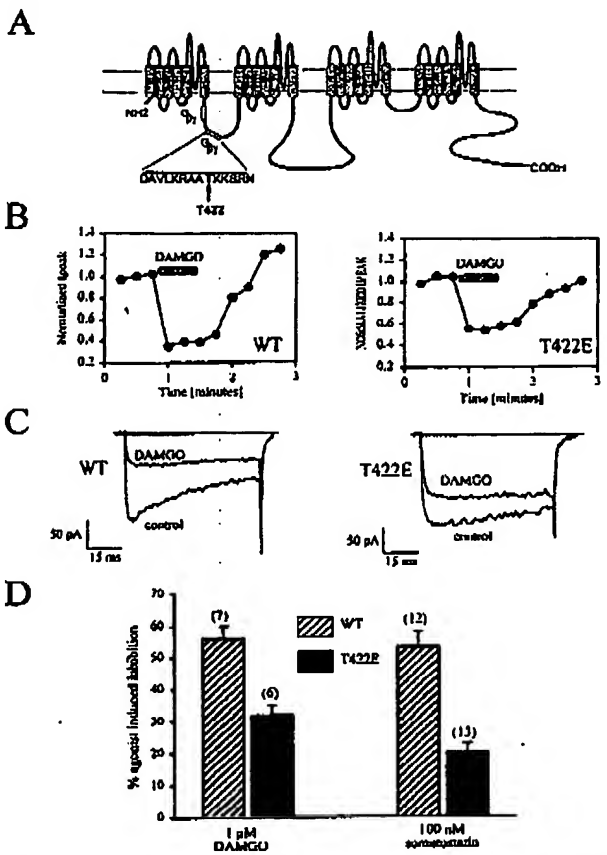


FIG. 1. **Cross-talk between G-protein modulation and PKC-dependent phosphorylation of N-type calcium channels.** *A*, schematic representation of the N-type calcium channel $\alpha_1$ subunit. The $\alpha_1$ subunit consists of four homologous domains that are linked by large intracellular loops (30). The cytoplasmic linker between domains I and II is one of the prime targets for binding of G$\beta\gamma$ subunits (12, 22). Residue Thr-422 forms the core of a protein kinase C consensus site (see amino acid sequence) which underlies G-protein PKC cross-talk (4). *B*, time course of $\mu$-opioid receptor-mediated G-protein inhibition of wild-type and mutant (T422E) N-type calcium channels. Cells were held at −100 mV and depolarized to +20 mV. Note that the effects are fully reversible. *C*, current records illustrating the effects of T422E mutation on $\mu$-opioid receptor-mediated G-protein inhibition of N-type calcium channels. The *solid line* indicates the base line. *D*, comparison of the effects of T422E mutation on $\mu$-opioid receptor- and somatostatin receptor-mediated inhibition of N-type calcium channels. The somatostatin data were taken from our previous work (4). Note that a mutation that permanently mimics phosphorylation of residue 422 affects the somatostatin receptor-mediated inhibition significantly more strongly than that induced by $\mu$-opioid receptor activation.

## RESULTS AND DISCUSSION

We have previously shown that activation of protein kinase C in tsa-201 cells reduces a somatostatin receptor-induced G-protein inhibition of N-type calcium channels (4). A point mutation in the domain I-II linker region of the channel (T422E) mimicked the effects of PKC activation, whereas a substitution to alanine precluded the effects of PKC on G-protein inhibition. We concluded that PKC/G-protein cross-talk occurs via phosphorylation of threonine 422 (4). To assess whether this effect was selective for somatostatin receptors, we coexpressed $\mu$-opioid receptors with wild type or mutant (T422E) N-type ($\alpha_{1B}$, $\beta_{1b}$, $\alpha_2$-$\delta$) calcium channels and applied 1 $\mu$M DAMGO to trigger G protein inhibition of the channels via the receptor. As seen in Fig. 1, wild type channels underwent a robust, reversible inhibition by 56 ± 4% in response to opioid receptor activation. In contrast, the T422E mutant exhibited a reduced degree of G-protein inhibition (32 ± 3%), in accord with our assertion that a negative charge (which permanently mimics phosphorylation) in position 422 reduces G protein efficacy. A comparison with our previous work with somatostatin receptors (4), however, shows that the somatostatin receptor-mediated inhibition of N-type calcium channels was significantly more strongly affected than the $\mu$-opioid response (20 ± 3% *versus* 32 ± 3%, $p < 0.05$), whereas the wild type channels were similarly inhibited by both pathways (DAMGO, 56 ± 4%; somatostatin, 53 ± 5%, $p > 0.05$). Although the latter observation indicates that both receptor types are equally effectively coupled to wild type N-type channels in tsa-201 cells, the observation with the T422E mutant suggests that there are nonetheless differences in the way the two receptor types couple to the channels. It is possible that the two receptor types couple to the

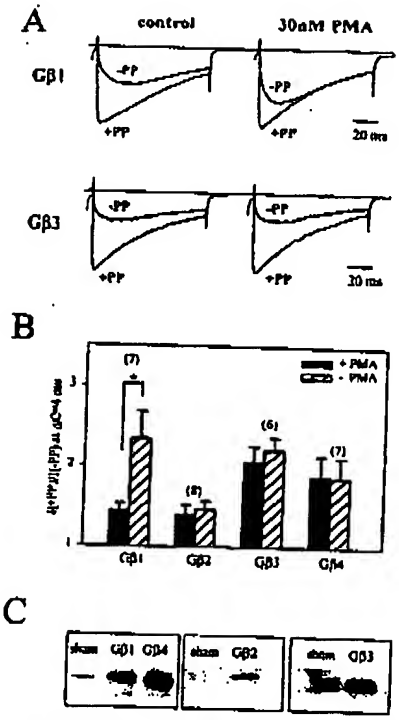


FIG. 2. *A*, current records illustrating the effects of PKC-dependent phosphorylation of N-type calcium channels on the degree of prepulse relief seen with either $G\beta_1$ or $G\beta_2$. The traces were arbitrarily scaled to overlap at the peak current levels obtained after the prepulse, and hence no legends for current levels are provided. Note that the degree of prepulse facilitation obtained with $G\beta_1$ becomes diminished following a 2-min application of 30 nM PMA. *B*, effect of 30 nM PMA on the degree of prepulse relief obtained with four types of G-protein $\beta$ subunits. In this experiment, a 50-ms prepulse to +150 mV was applied, followed by a 4-ms repolarization to −100 mV and a test pulse to +20 mV. Note that PMA selectively affects $G\beta_1$-mediated responses. *Error bars* are standard errors, the *asterisk* denotes significance at the 0.05 level (paired *t* test). *C*, Western blot analysis of cell lysate obtained from either sham-transfected tsa-201 cells or from tsa-201 cells transfected with one of four G-protein $\beta$ subunits. The exogenous expression of either of the four $G\beta$ subunits results in a substantial increase in $G\beta$ levels despite the notion that our transfection efficiency is typically around 25%. The blots for $G\beta_1$ and $G\beta_4$ were probed with an antibody that recognizes both of these G-protein subtypes.

channels via different G-protein heterotrimer compositions, which may be differentially affected by the presence of the T422E mutation.

The key G-protein species involved in direct inhibition of N-type calcium channels is the $G\beta$ subunit (5, 6, 16, 18). To date, five different types of $G\beta$ subunits have been identified in mammalian brain (17), and we have recently shown that N-type channels expressed in tsa-201 cells are most effectively inhibited by $G\beta_1$ and $G\beta_3$, whereas $G\beta_4$ and $G\beta_2$ mediate a somewhat smaller inhibition, and $G\beta_5$ is ineffective and is thus not further considered here (16).

To test the possibility that PKC/G-protein cross-talk might preferentially affect a subset of the $G\beta$ subunit isoforms, we coexpressed wild type channels with one of four different $G\beta$ subtypes ($+G\gamma_2$) and used a strong depolarizing prepulse to compare the resulting tonic G-protein inhibition before and after phosphorylation of the channel by protein kinase C (elicited by 2-min application of 30 nM PMA). As shown in Fig. 2, in cells expressing $G\beta_1$, the degree of prepulse relief became reduced from 2.4 ± 0.3 to 1.4 ± 0.1 ($n = 7$, $p < 0.02$, paired *t* test) following PMA treatment, whereas inhibition by the three other G-protein $\beta$ subunit subtypes was not significantly altered (Fig. 2*B*). Thus, these data suggest that PKC/G-protein cross-talk selectively affects $G\beta_1$-mediated responses.

Although N-type calcium channels expressed in tsa-201 cells show only negligible background G-protein inhibition in the absence of exogenous $G\beta\gamma$ (16), it is true that these cells contain endogenous G protein $\beta\gamma$ subunits that are presumably complexed as $\alpha\beta\gamma$ heterotrimers. To assess the likelihood of contamination of our results by endogenously present $G\beta$ proteins, we carried out Western blot analysis of control cells and cells transfected with either one of the four $G\beta$ subunits. As seen in Fig. 2*C*, exogenous expression of each of the four subtypes tested resulted in a substantial increase in $G\beta$ levels compared with those found in control cells, confirming that the exogenously expressed subunits are the predominant G-protein species in transfected cells.

To obtain an indication of the change in affinity of the channels for the G-proteins, which occurs after phosphorylation, we utilized dynamic prepulse protocols to determine the time constants of recovery from inhibition (by varying prepulse duration) and reinhibition after the prepulse (by varying the duration between the prepulse and the test pulse, see Fig. 3*A*). However, after prolonged application of PMA (>3 min), we noted that current levels began to run down, which would interfere with the accuracy of these types of experiments. Hence, we chose to utilize the T422E mutant for our kinetic measurements.

Fig. 3*B* shows that the T422E mutant behaved like the phosphorylated wild type channel with regard to the $G\beta$ subtype dependence of cross-talk, such that the mutation induced a selective decrease in the degree of prepulse relief seen in the presence of $G\beta_1$ (wild type, 2.8 ± 0.3; T422E, 1.9 ± 0.1; $p < 0.05$) whereas the facilitation ratios of the three remaining G-protein $\beta$ subunit isoforms did not differ significantly from those obtained with the wild type channels (note, however, that the data shown in Figs. 2*B* and 3*B* were obtained by slightly different protocols, and hence, absolute values for the degree of prepulse relief are not directly comparable). As seen in Fig. 3*C*, the T422E mutation selectively reduced the time constant of recovery from $G\beta_1$ inhibition (wild type, 11.0 ± 0.6 ms; T422E, 8.0 ± 0.6 ms; $p < 0.05$), indicating that one of the consequences of the T422E mutation is a slight destabilization of the $G\beta_1$-channel complex. In addition, the time course of $G\beta_1$ reinhibition after the prepulse (Fig. 3*D*) was slowed 3-fold as a result of the T422E mutation (wild type, 18.5 ± 2.4 ms; T422E, 51.7 ± 4.7 ms; $p < 0.05$). Interestingly, a small increase in the time course of reinhibition was also observed with $G\beta_2$ (WT, 46.2 ± 6.3 ms; T422E, 27.8 ± 4.1 ms; $p < 0.05$), indicating that the T422E mutation may exert subtle effects on this subunit. Overall, however, the data shown in Figs. 2 and 3 indicate that a mutagenically phosphorylated threonine residue in position 422 affects predominantly the inhibition of N-type calcium channels by $G\beta_1$ subunits.

To date, little is known about the association of individual types of seven helix transmembrane receptors with specific subsets of G-protein subunits. It has been shown, however, that antisense depletion of $G\beta_1$ subunits abolishes somatostatin receptor signaling in rat pituitary $GH_3$ cells (22). It is thus likely that somatostatin receptors exclusively couple to $G\beta_1$ subunits, which can account nicely for our observation that the T422E mutation dramatically reduced the G-protein inhibition induced by both overexpression of $G\beta_1$ and upon activation of somatostatin receptors. In contrast, it is possible that the $\mu$-opioid receptor couples to N-type calcium channels through more than one type of G-protein $\beta$ subunit. Whereas the observation that the opioid response was reduced in the T422E mutant would suggest that at least part of the opioid signaling

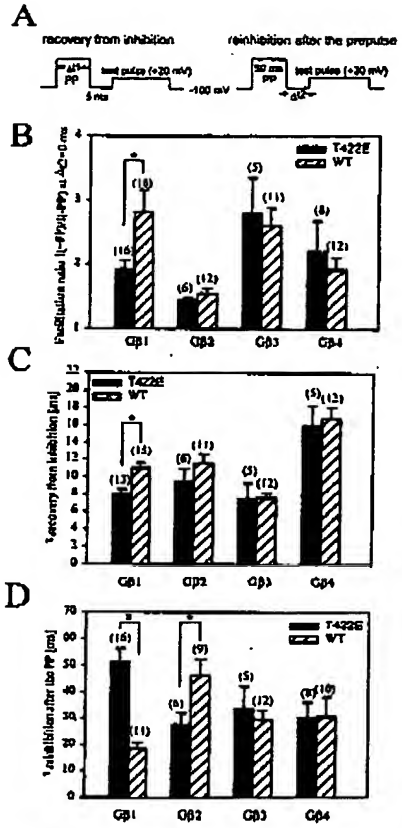


FIG. 3. **Dependence of PKC/G-protein cross-talk on the Gβ subunit isoform.** The wild type data were taken from our previous work (16). *A*, prepulse paradigms used to determine the kinetics of removal of G-protein inhibition during the prepulse (*i.e.* recovery, *left panel*), and those of reinhibition following the prepulse (*PP*) (*right panel*). *B*, degree of G-protein inhibition of wild type and T422E mutant N-type calcium channels in form of facilitation ratios and as a function of G-protein β subunit isoform. The facilitation ratios (I + PP)/I(−PP) were obtained as described under "Experimental Procedures" and in Arnot *et al.* (16), which relies on back extrapolation of the data to $t_2 = 0$ ms (hence the difference in absolute facilitation levels compared with Fig. 2*B*). Note that the T422E mutation selectively affects $G\beta_1$-mediated inhibition. *C*, the T422E mutation selectively increases the rate of development of facilitation for the $G\beta_1$ subtype. *D*, T422E reduces the rate of G-protein reinhibition for $G\beta_1$ and $G\beta_2$ but does not affect the reinhibition rates observed with $G\beta_3$ and $G\beta_4$.

is mediated by $G\beta_1$, our data showing that the opioid and somatostatin responses were not equally affected by the T422E substitution (Fig. 1*D*), however, support a mechanism in which μ-opioid receptors may couple to a mixed population of G-protein β subunit isoforms. This scenario would account for the intermediate response we observed.

The data shown in Fig. 3, *C* and *D*, provide some insights into the molecular mechanisms by which cross-talk may occur. It is now widely accepted that Gβγ physically interacts with the N-type calcium channel domain I-II linker region (8, 23, 24), thus phosphorylation events or amino acid substitutions occurring in this region may affect the binding interactions between the channel and the Gβγ dimers. Consistent with such an effect, the T422E substitution resulted in a significant decrease in the time constant for development of facilitation during the prepulse and an increase in the time constant for reinhibition after the prepulse. In view of evidence that G proteins must physically dissociate from the channel during the prepulse (8), these changes in kinetics likely reflect a change in the G-protein association and dissociation kinetics, and thus an overall reduction in the affinity of the channel for $G\beta_1$, which may also account for the reduction in the degree of prepulse relief seen with the T422E mutant. Our experiments do not permit us to provide an absolute value for the PKC-induced changes in the equilibrium dissociation constant between the G proteins and the channels, because the kinetics for recovery from inhibition during the prepulse (+150 mV) and reinhibition after the prepulse (*i.e.* repolarization to −100 mV) were obtained at different voltages. Nonetheless, based on the 3-fold decrease in reinhibition kinetics and the 1.4-fold speeding of the recovery time constant, we estimate that the presence of the T422E mutation may perhaps result in an ~5-fold change in $G\beta_1$ affinity.

An important aspect to consider is control over the G protein expression levels in our experiments. Our Western blot analysis shows that each of the four Gβ subtypes expressed well in tsa-201 cells, indicating that the exogenously expressed G-protein subunits are much more abundant than endogenously present Gβ, although it is difficult to predict relative expression levels among the four Gβ subtypes from Western blots as antibody sensitivity may vary. In the experiments shown in Fig. 2*B*, the inhibition of nonphosphorylated and phosphorylated channels by each Gβ subtype was studies in the same cell, and hence, at a constant Gβ concentration. In the experiments shown in Fig. 3*B*, the effects of each Gβ subtype on wild type and mutant channels needed was assessed in different cells. However, for each given Gβ subtype, wild type and mutant channels were studied under identical conditions, thus attributing any changes in channel inhibition to residue 422 rather than Gβ levels. Both types of experiments resulted in essentially the same result, namely that only $G\beta_1$-mediated responses were affected by phosphorylation/mutation of the channel.

Although *in vivo* evidence supporting our conclusions is still lacking, it is tempting to speculate about the implications of observations for neurotransmission: calcium influx through N-type and P/Q-type calcium channels is essential for the fast release of neurotransmitter (25). Regulation of presynaptic calcium channel activity by cytoplasmic messenger molecules is thus an essential means for the precise control of neurotransmission. The activation of opioid receptors, for example, depresses synaptic activity (13, 26), and tonic G-protein inhibition at presynaptic nerve termini contributes to paired pulse facilitation (27–29). We have previously presented evidence that integration of protein kinase C and Gβγ pathways by the N-type calcium channel $\alpha_1$ subunit can produce multiple levels of current activity (4). If our results are extrapolated to an *in vivo* situation, the notion that the extent of this integration appears to depend on the type of Gβ subunit isoform present may provide additional avenues by which neurotransmitter receptors may regulate calcium homeostasis in the presynapse. Together with the notion that different types of G-protein β subunits do not only affect N-type channel activity to different degrees (16–18) but also show pronounced differences in their abilities to inhibit P/Q-type calcium channels (16), the unique activation of specific Gβγ combinations by different types of seven-helix transmembrane receptors could provide a highly complex regulatory mechanism for the fine tuning of presynaptic calcium levels and, thus, neurotransmitter release.

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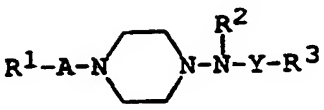
(54) Title: NEW USE OF AMINOPIPERAZINE DERIVATIVES

(57) Abstract

This invention relates to the use of a compound of formula (I) wherein each symbol is as defined in the description, or its pharmaceutically acceptable salt, for treating and/or preventing schizophrenia, depression, stroke, and the like.

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DESCRIPTION

# NEW USE OF AMINOPIPERAZINE DERIVATIVES

## TECHNICAL FIELD

This invention relates to a new use of aminopiperazine derivatives and pharmaceutically acceptable salts thereof for the treatment and/or prevention of schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism in mammals.

## BACKGROUND ART

The aminopiperazine derivatives used in this invention are known as described in PCT International Publication No. WO 91/01979 that said aminopiperazine derivatives possess the potentiation of the cholinergic activity and are useful in the treatment of disorders in the central nervous system for human beings, and more particularly in the treatment of amnesia, dementia, senile dementia and the like.

## DISCLOSURE OF INVENTION

The present invention relates to a new use of aminopiperazine derivatives and pharmaceutically acceptable salts thereof for the treatment and/or prevention of schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism for mammals.

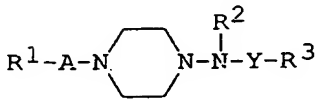
Accordingly, this invention is to provide a new use of aminopiperazine derivatives and pharmaceutically acceptable salts thereof for treating and/or preventing schizophrenia,

depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism.

Further, this invention is to provide an agent and a pharmaceutical composition for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism, which comprises said aminopiperazine derivatives and pharmaceutically acceptable salt thereof.

Still further, this invention is to provide a therapeutic method for the treatment and/or prevention of schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism, which comprises administering said aminopiperazine derivatives and pharmaceutically acceptable salts thereof to mammals.

The aminopiperazine derivatives used in this invention can be represented by the following general formula [I] :



[I]

wherein $R^1$ is lower alkyl, aryl, ar(lower)alkoxy or a heterocyclic group, each of which may be substituted with halogen,

$R^2$ is hydrogen or lower alkyl,

$R^3$ is cyclo(lower)alkyl, aryl or ar(lower)alkyl, each of which may be substituted with halogen,

A is $-\overset{O}{\overset{\|}{C}}-$, $-SO_2-$ or lower alkylene, and

Y is $-\overset{O}{\overset{\|}{C}}-$, $-SO_2-$ or $-\overset{O}{\overset{\|}{C}}NH-$,

and pharmaceutically acceptable salts thereof.

Said compound (I) and pharmaceutically acceptable salts thereof are useful in the treatment and/or prevention of schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism in mammals.

Particulars of the various definitions mentioned in this specification and preferred examples thereof are explained in the following.

The term "lower" is intended to mean a group having 1 to 6 carbon atom(s), unless otherwise provided.

Suitable "lower alkyl" may be a straight or branched one such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl, hexyl, or the like, in which preferable one is methyl.

Suitable "aryl" may be phenyl, naphthyl, tolyl, xylyl, mesityl, cumenyl, and the like, in which preferable one is phenyl or naphthyl.

Suitable "ar(lower)alkoxy" may be benzyloxy, phenethyloxy, phenylpropoxy, benzhydryloxy, trityloxy and the like.

Suitable "heterocyclic group" may include saturated or unsaturated, monocyclic or polycyclic one containing at least one hetero atom such as nitrogen atom, oxygen atom or sulfur atom.

The preferred examples of thus defined "heterocyclic group" may be an unsaturated, 3 to 8-membered, more preferably 5 or 6-membered heteromonocyclic group containing 1 to 4-nitrogen atom(s), for example, pyrrolyl, imidazolyl, pyrazolyl, pyridyl, pyridyl N-oxide, dihydropyridyl, tetrahydropyridyl, pyrimidyl, pyrazinyl, pyridazinyl, triazinyl, triazolyl, tetrazinyl, tetrazolyl, etc.;

unsaturated, condensed heterocyclic group containing 1 to 5 nitrogen atom(s), for example, indolyl, isoindolyl, indolizinyl, benzimidazolyl, quinolyl, isoquinolyl, indazolyl, benzotriazolyl, etc.;

unsaturated, 3 to 8-membered heteromonocyclic group containing 1 to 2 oxygen atom(s) and 1 to 3 nitrogen atom(s), for example, oxazolyl, isoxazolyl, oxadiazolyl, etc.;

saturated, 3 to 8-membered heteromonocyclic group containing 1 to 2 oxygen atom(s) and 1 to 3 nitrogen atom(s), for example, morpholino, sydnonyl, etc.;

unsaturated, condensed heterocyclic group containing 1 to 2 oxygen atom(s) and 1 to 3 nitrogen atom(s), for example, benzoxazolyl, benzoxadiazolyl, etc.;

unsaturated, 3 to 8-membered heteromonoyclcic group containing 1 to 2 sulfur atom(s) and 1 to 3 nitrogen atom(s), for example, thiazolyl, isothiazolyl, thiadiazolyl, etc.;

unsaturated, 3 to 8-membered heteromonocyclic group containing 1 to 2 sulfur atom(s), for example, thienyl, etc.;

unsaturated, condensed heterocyclic group containing 1 to 2 sulfur atom(s) and 1 to 3 nitrogen atom(s), for example, benzothiazolyl, benzothiadiazolyl, etc.;

unsaturated, 3 to 8-membered heteromonocyclic group containing an oxygen atom, for example, furyl, etc.;

unsaturated, condensed heterocyclic group containing 1

to 2 sulfur atom(s), for example, benzothienyl, etc.;

unsaturated, condensed heterocyclic group containing 1 to 2 oxygen atom(s), for example, benzofuranyl, etc.; or the like.

Suitable "cyclo(lower)alkyl" may be cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like.

Suitable "ar(lower)alkyl" may be benzyl, phenethyl, phenylpropyl, benzhydryl, trityl, and the like.

Suitable "lower alkylene" may be methylene, ethylene, propylene, pentamethylene, hexamethylene, and the like.

The above-mentioned "lower alkyl", "aryl", "ar(lower)alkoxy", "heterocyclic group", "cyclo(lower)alkyl" and "ar(lower)alkyl" may be substituted with halogen [e.g. fluorine, chlorine, bromine and iodine].

Preferred compound [I] is one which has a lower alkyl, phenyl, naphthyl or thienyl for $R^1$, hydrogen or lower alkyl for $R^2$, phenyl which may be substituted with a halogen for $R^3$, $-\overset{O}{\overset{\|}{C}}-$ for A, and $-\overset{O}{\overset{\|}{C}}-$ or $-SO_2-$ for Y.

More preferred compound [I] is one which has a lower alkyl for $R^1$, hydrogen for $R^2$, phenyl which is substituted with a halogen for $R^3$, $-\overset{O}{\overset{\|}{C}}-$ for A, and $-\overset{O}{\overset{\|}{C}}-$ for Y.

Most preferred compound [I] is N-(4-acetyl-1-piperazinyl)-4-fluorobenzamide.

Suitable pharmaceutically acceptable salts of the compound [I] are conventional non-toxic salts and include acid addition salt such as an inorganic acid addition salt [e.g. hydrochloride, hydrobromide, sulfate, phosphate, etc.], an organic acid addition salt [e.g. formate, acetate, trifluoroacetate, maleate, tartrate, methanesulfonate,

benzenesulfonate, toluenesulfonate, etc.], a salt with an amino acid [e.g. aspartic acid salt, glutamic acid, salt, etc.] and the like.

It is to be noted that the compound [I] may include one or more stereoisomer(s) due to asymmetric carbon atoms, and all of such isomers and mixture thereof are included within the scope of this invention.

Additionally, it is to be noted that any hydrate of the compound [I] is also included within the scope of this invention.

Now in order to show the utility of the compound [I] and pharmaceutically acceptable salts thereof in this invention, the pharmacological test was carried out and its abstract is shown in the following.

The effect of the compound [I] upon cognitive function was examined using an operant delayed non-match to place paradigm (DNMTP) task which is shown to be disrupted dose-dependently by the administration of haloperidol. The following interactions were explored: haloperidol plus amphetamine, haloperidol plus the compound [I] and haloperidol plus the compound [I] and amphetamine. Neither a low dose of amphetamine nor two doses of the compound [I] when administered with haloperidol, or alone, altered the profile of performance relative to control. The experiments with haloperidol and the compound [I] plus amphetamine revealed a profound attenuation of the deficits associated with incerasing doses of haloperidol by the larger dose of the compound [I].

These expeiments confirmed that the compound [I] has a specific effect on dopaminergic status which appears to be state dependent and is useful for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine

withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism.

For therapeutic purpose, the compound [I] and a pharmaceutically acceptable salt thereof of the present invention can be used in a form of pharmaceutical preparation containing one of said compounds, as an active ingredient, in admixture with a pharmaceutically acceptable carrier such as an organic or inorganic solid or liquid excipient suitable for oral or parenteral administration. The pharmaceutical preparations may be capsules, tablets, dragees, granules, solution, suspension, emulsion, or the like. If desired, there may be included in these preparations, auxiliary substances, stabilizing agents, wetting or emulsifying agents, buffers and other commonly used additives.

While the dosage of the compound [I] will vary depending upon the age and condition of the patient, an average single dose of about 0.1 mg, 1 mg, 10 mg, 50 mg, 100 mg, 250 mg, 500 mg and 1000 mg of the compound [I] may be effective for treating the above-mentioned diseases. In general, amounts between 0.1 mg/body and about 1,000 mg/body may be administered per day.

The following Examples is given for the purpose of illustrating this invention.

Example 1

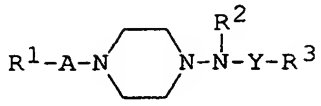
(Capsule)

| | |
|---|---|
| N-(4-Acetyl-1-piperazinyl)-4-fluorobenzamide | 5 mg |
| Lactose | 80 mg |

The above-mentioned ingredients were mixed and the mixture was encapsulated to provide the capsule.

CLAIMS

1. Use of a compound of the formula :



[I]

wherein $R^1$ is lower alkyl, aryl, ar(lower)alkoxy or a heterocyclic group, each of which may be substituted with halogen,

$R^2$ is hydrogen or lower alkyl,

$R^3$ is cyclo(lower)alkyl, aryl or ar(lower)alkyl, each of which may be substituted with halogen,

A is $-\overset{O}{\overset{\|}{C}}-$, $-SO_2-$ or lower alkylene, and

Y is $-\overset{O}{\overset{\|}{C}}-$, $-SO_2-$ or $-\overset{O}{\overset{\|}{C}}NH-$,

or its pharmaceutically acceptable salt for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism.

2. A use of the compound defined in Claim 1 as an agent for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity

disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism.

3. An agent for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism, which comprises the compound defined in Claim 1.

4. A method for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism, which comprises administering the compound defined in Claim 1 to mammals.

5. A use of the compound defined in Claim 1 for manufacturing a medicament for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's disease or autism.

6. A pharmaceutical composition for treating and/or preventing schizophrenia, depression, stroke, head injury, nicotine withdrawal, spinal cord injury, anxiety, pollakiuria, incontinence of urine, myotonic dystrophy, attention deficit hyperactivity disorder, excessive daytime sleepiness (narcolepsy), Parkinson's

disease or autism, which comprises the compound defined in Claim 1 in admixture with a carrier or excipient.

7. A process for preparing the pharmaceutical composition of Claim 6, which is characterized by admixing the compound with a carrier or excipient.

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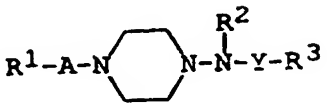


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| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
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# INTERNATIONAL SEARCH REPORT

Intern. ial Application No
PCT/JP 97/04704

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/495

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | N. MATSUOKA ET AL.: "Effect of FR121196, a Novel Cognitive Enhancer, on the Memory Impairment of Rats in Passive Avoidance and Radial Arm Maze Tasks" J. PHARMACOL. EXP. THER., vol. 263, no. 2, November 1992, pages 436-444, XP002058603 see the whole document --- | 1-7 |
| X | EP 0 436 734 A (FUJISAWA PHARMACEUTICAL CO., LTD.) 17 July 1991 cited in the application see page 6, line 54 - page 7, line 6 & WO 91 01979 A (FUJISAWA PHARMACEUTICAL CO., LTD.) cited in the application --- -/-- | 3,6,7 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 March 1998 | 1 0. 07. 98 |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Authorized officer<br>THEUNS, H |

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# INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/JP 97/04704

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | M. YAMAZAKI ET AL.: "FK960 N-(4-Acetyl-1-piperazinyl)-p-fluorobenzamide monohydrate Ameliorates the Memory Deficits in Rats through a Novel Mechanism of Action" J. PHARMACOL. EXP. THER., vol. 279, no. 3, December 1996, pages 1157-1173, XP002058604 see the whole document --- | 1-7 |
| X | N. MAEDA ET AL.: "Involvement of Raphe-Hippocampal Serotonergic and Septo-Hippocampal Cholinergic Mechanisms in the Penile Erection Induced by FR121196, a Putative Cognitive Enhancer" JPN. J. PHARMACOL., vol. 68, no. 1, May 1995, pages 85-94, XP002058605 see page 93, left-hand column --- | 1-7 |
| X | M.YAMAZAKI ET AL.: "FR121196, a Potential Antidementia Drug, Ameliorates the Impaired Memory of Rat in the Morris Water Maze" J.PHARMACOL. EXP. THER., vol. 272, no. 1, January 1995, pages 256-263, XP002058606 see abstract ----- | 1-7 |

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP 97/04704

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Group 1. Claims 1-7 (all partially)
Group 2. Claims 1-7 (all partially)
Group 3. Claims 1-7 (all partially)
Group 4. Claims 1-7 (all partially)
Group 5. Claims 1-7 (all partially)
Group 6. Claims 1-7 (all partially)
Group 7. Claims 1-7 (all partially)
Group 8. Claims 1-7 (all partially)
Group 9. Claims 1-7 (all partially)

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Group 1. Claims 1-7

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
PCT/JP 97/04704

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP 0436734 A | 17-07-91 | DE 69022965 D | 16-11-95 |
| | | DE 69022965 T | 04-04-96 |
| | | DK 436734 T | 20-11-95 |
| | | HK 64196 A | 19-04-96 |
| | | WO 9101979 A | 21-02-91 |
| | | JP 2531304 B | 04-09-96 |
| | | US 5250528 A | 05-10-93 |

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